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Superoxide Dismutase, Catalase and Glutathione Peroxidase Activities in Human Blood: Influence of Sex, Age and Cigarette Smoking

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Objective: To obtain reference ranges for each of the main antioxidant enzymes (AOE) and analyze the influence of sex, age, and cigarette smoking on AOE activity in human blood.

Design and Methods: We investigated superoxide dismutase (SOD), catalase (CAT), and seleno-dependent glutathione peroxidase (GSH-Px) activities in the whole blood from 103 healthy subjects, from 18–67 years old (51 males and 52 females).

Results: We found a large and highly significant interindividual variability in the activity of all the AOE studied ($p < 0.001$). The interindividual coefficients of variation were 13.5% for SOD, 21.0% for CAT, and 36.2% for GSH-Px, indicating that GSH-Px exhibits the highest interindividual variability. Females showed higher SOD ($p < 0.001$) and CAT ($p < 0.001$) activities but lower GSH-Px ($p < 0.05$) activity than males. We found a significant effect of age on SOD activity ($p < 0.001$), showing that in human blood it decreases with age and that this decrease is not linear, beginning at 28 years of age. We also observed a linear effect of age on GSH-Px activity indicating that the activity of this enzyme increases with age ($p < 0.01$). No effect of age on CAT activity was observed ($p > 0.05$). AOE activity in smokers was found not to be significantly different from that observed in non-smokers ($p > 0.05$) except in the case of CAT activity in females, which was found to be lower in smokers than in non-smokers ($p < 0.05$). In addition, we determined reference ranges for the activity of each antioxidant enzyme studied.

Conclusions: Our results confirm that AOE activity in human blood exhibits a wide interindividual variability and suggest that this variability may be ascribed, at least in part, to the sex and age of the individuals. Moreover, our results suggest that cigarette smoking does not influence AOE activity in human blood. Accordingly, it is suggested that for clinical purposes it may be necessary to consider the sex and age of the subjects involved in the study.

KEY WORDS: antioxidant enzymes; superoxide dismutase; catalase; glutathione peroxidase; human blood; aging; cigarette smoking.

Introduction

Oxygen free radicals are highly reactive species that can cause a wide spectrum of cell damage including lipid peroxidation, enzymes inactivation and DNA damage (1,2). Mammalian cells are protected against free radicals by enzymatic and non-enzymatic antioxidant defenses (1,2). The primary antioxidant enzymes (AOE) are superoxide dismutase (SOD; EC 1.15.1.1), which catalyzes the dismutation of superoxide anions ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) and catalase (CAT; EC 1.11.1.6) and seleno-dependent glutathione peroxidase (GSH-Px; EC 1.11.1.9), which catalyze the degradation of H_2O_2 to H_2O and O_2. GSH-Px catalyzes the reduction of H_2O_2 to water at the expense of reduced glutathione (GSH) (1).

During the past 25 years, AOE activity in normal and tumor cells from animals and human beings has received increasing attention. Indeed, it has been pointed out that free radicals and hence, antioxidant enzymes, play a role in several human diseases such as rheumatoid arthritis, Alzheimer's disease, cancer, uremia, etc. (3–10). Free radicals and AOE have been also assumed to be causal in aging (11). Moreover, it has been reported that the sensitivity of human lymphocyte chromosomes to ionizing radiation and bleomycin is inversely correlated with SOD levels in blood (7,12–14) and that AOE activity in whole blood is directly correlated with AOE activity in erythrocytes (14). Thus, measurement of SOD activity in whole blood or erythrocytes could be useful to predict the chromosomal sensitivity of human lymphocytes to bleomycin and ionizing radiation. Due to these findings, the determination of AOE activity in blood and different tissues appeared to be of clinical interest. There are several previous reports regarding AOE activity in human erythrocytes from normal individuals as well as cancer patients (15–21). However, in regard to healthy subjects, most of the studies involved a restricted population and considered only one or two of the main AOE. Complete understanding of the AOE system in human blood as a function of sex, age, and other factors that could influence AOE activity requires measurement of all the primary AOE and not just measurement of one or two of these enzymes, as previously reported. Surprisingly, the cellular anti-

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oxidant enzyme system has not been systematically studied in human blood with respect to sex, age, or cigarette smoking in healthy subjects. Accordingly, in this report, by using three simple and sensitive assays suitable for clinical determinations, we measured the activities of all the main AOE, *i.e.*, SOD, CAT, and GSH-Px in whole blood from healthy subjects, in order to obtain reference ranges for each AOE and analyze the influence of sex, age, and cigarette smoking on AOE activity.

Methods

Subjects

We assayed SOD, CAT, and GSH-Px activities in whole blood from 103 healthy subjects aged 18–67 years (51 males and 52 females); 35 of them were smokers. Blood samples were obtained from students or employees of the University of La Plata (Buenos Aires Province, Argentina). None of the subjects had any known pathologies at the time of sampling. Subjects fasted after midnight before blood collection the next morning. The present study was authorized by the Dirección de Sanidad (Health Department) from the University of La Plata.

Blood lysates preparation

We collected 1.0 mL of peripheral blood in heparinized syringes. We removed 0.2 mL aliquots to determine the hemoglobin (Hb) concentration with a standard kit (HemogloWiener, Lab. Wiener, Rosario, Argentina) involving the cyanmethemoglobin method (Drabkin's method). The remaining blood was diluted in 9 g/L NaCl solution (dilution 1:10) and lysed with 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA) plus sonication (W-225 R Sonicator, Heat Systems, Ultrasonics Inc., Plainview, L.I., New York, NY, USA) using four bursts of 15 s each while kept on ice. Cell membranes were removed by centrifugation at 12000 × *g* for 5 min at 4 °C and the supernates were used for determining AOE activity. The Hb concentration was also determined in the hemolysates, which were then stored in liquid nitrogen until enzyme assays. AOE activity of blood samples was measured within 1 week of sampling. In a previous work we found that a strong positive correlation exists between AOE activity in whole blood and AOE activity in human erythrocytes (14). Due to this previous finding, we decided to perform enzyme assays in whole blood.

Enzyme assays

Enzymatic determination of SOD

SOD activity was measured by the method of Sun *et al.* (22,23) which consists of the inhibition of nitroblue tetrazolium (NBT) reduction with xanthine-xanthine oxidase used as a superoxide generator. One unit of SOD activity is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. Enzyme activity was expressed as units per mg of Hb (U/mgHb).

Enzymatic determination of CAT

CAT activity was determined according to Lück (24). One unit of CAT is defined as the amount of enzyme which liberates half the peroxide oxygen from a H_2O_2 solution in 100 s at 25 °C. Enzyme activity was expressed as units per mg of Hb (U/mgHb).

Enzymatic determination of GSH-Px

We measured GSH-Px activity by a modification of the coupled assay procedure of Paglia and Valentine (25) as described by Lawrence and Burk (26) using hydrogen peroxide as substrate. Hb was converted to cyanmethemoglobin by reaction with potassium cyanide and potassium ferricyanide to minimize its pseudoperoxidase reactivity according to Günzler *et al.* (27). GSSG produced by the action of GSH-Px and hydrogen peroxide was reduced by GSSG-RD (glutathione reductase) and NADPH, the decrease in concentration of NADPH being recorded at 340 nm. The reaction mixture, maintained at 25 °C, consisted of 0.05 M potassium phosphate buffer (pH 7.0), 1 mM EDTA (Sigma Chemical Co.), 1 mM NaN_3 (to block CAT activity in the sample), 1 mM GSH (Sigma Chemical Co.), 1 U/mL yeast GSSG-RD (Type III; Sigma Chemical Co.), 0.2 mM NADPH (Sigma Chemical Co.) and 0.25 mM hydrogen peroxide, in a total volume of 1 mL. The assay kinetics were calculated by using a millimolar extinction coefficient for NADPH of 6.22 $cm^2/\mu mol$ at 340 nm. Enzyme activity was expressed as milliunits per mg of Hb (mU/mgHb).

Enzyme assays of each sample were performed in duplicate on a Lambda 3B UV/VIS Spectrophotometer (Perkin-Elmer Corporation, Norwalk, CT, USA).

Data analyses

The interindividual variability in AOE activity, the contribution of the experimental error to the total variability observed and the effects of sex, age, and cigarette smoking on AOE activity were analyzed by one-way analysis of variance (ANOVA) with an IBM PC by using the Statgraphics software 6.0 (Statistical Graphics Corporation, Rockville, Maryland, USA). The level of significance chosen was $p < 0.05$.

Results

Table 1 shows the mean values of SOD, CAT, and GSH-Px activities in whole blood and the interindividual coefficients of variation for each enzyme. One-way ANOVA showed a statistical significant interindividual variability in the activity of all AOE studied ($F_{102,102}$ = 14.49 for SOD, 31.12 for CAT and

TABLE 1
Antioxidant Enzymes Activity in Human Blood
(mean ± SD)

Gender (sample size)	SOD (U/mgHb)	CAT (U/mgHb)	GSH-Px (mU/mgHb)
Males + Females (103)	12.1 ± 1.7	9.2 ± 1.9	0.3 ± 0.1
Range	(7.8–17.6)	(4.1–14.6)	(0.1–0.7)
Coeff. Variation	13.5%	21.0%	36.2%
Males (51)	11.8 ± 1.5	8.4 ± 1.7	0.4 ± 0.1
Range	(8.5–16.1)	(4.1–13.8)	(0.1–0.7)
Coeff. Variation	12.2%	19.7%	35.5%
Females (52)	12.5 ± 1.8[a]	10.0 ± 1.9[a]	0.3 ± 0.1[b]
Range	(7.8–17.6)	(5.8 ± 14.6)	(0.1–0.7)
Coeff. Variation	14.2%	18.6%	35.8%

SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase.
[a] $p < 0.001$
[b] $p < 0.05$

7.25 for GSH-Px, $p < 0.001$). No statistical differences between duplicates of each sample were found ($p > 0.05$), the mean values being 12.1 ± 1.7 and 12.1 ± 1.7 U/mgHb for SOD, 9.1 ± 1.9, and 9.2 ± 2.0 U/mgHb for CAT and 0.3 ± 0.1 and 0.3 ± 0.1 mU/mgHb for GSH-Px. Accordingly, it is valid to assume that the interindividual differences observed in AOE blood levels have a biological meaning. The interindividual coefficients of variation for each AOE studied were 13.5% for SOD, 21.0% for CAT, and 36.2% for GSH-Px (Table 1). Females showed higher SOD ($F_{1,102} = 66.81, p < 0.001$) and CAT ($F_{1,102} = 572.41, p < 0.001$) activities but lower GSH-Px ($F_{1,102} = 26.32, p < 0.05$) activity than males (Table 1).

Table 2 shows the reference ranges of AOE activity in human blood calculated using the mean ± 2 SD interval. It should be noted that these calculated ranges are only valid for the enzymatic assays used.

We found a significant effect of the age of donors on SOD activity ($p < 0.001$) showing that SOD levels decrease with age and that this decrease is not linear, beginning at 28 years of age (Figure 1). We also found a linear effect of age on GSH-Px activity, showing that the activity of this enzyme increases with age ($p < 0.01$) (Figure 2). On the other hand, no effect of age on CAT activity was observed (Figure 3). Moreover, one-way ANOVA showed no significant differences in AOE activity between smokers and non-smokers ($p > 0.05$, Table 3), except in the case of CAT activity in females, which was found to be lower in smokers than in non-smokers ($p < 0.05$, Table 3).

TABLE 2
Reference Ranges of Antioxidant Enzymes Activity in Human Blood (mean ± 2 SD)

Enzyme	Males	Females
SOD (U/mgHb)[a]	9.0–14.6	9.0–15.9
CAT (U/mgHb)[b]	5.1–11.6	6.3–13.6
GSH-Px (mU/mgHb)[c]	0.1–0.6	0.1–0.5

SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase.
[a]Using the assay of Sun *et al.* (22).
[b]Using the assay of Lück (24).
[c]Using the assay of Lawrence and Burk (26).

Discussion

There are several previous reports regarding SOD, CAT and GSH-Px activities in human erythrocytes (4,8,15–21). However, in regard to healthy subjects, most of the studies involved a restricted population and considered only one or two of the AOE mentioned above. In this report, we measured the activities of all the main AOE, *i.e.*, SOD, CAT,

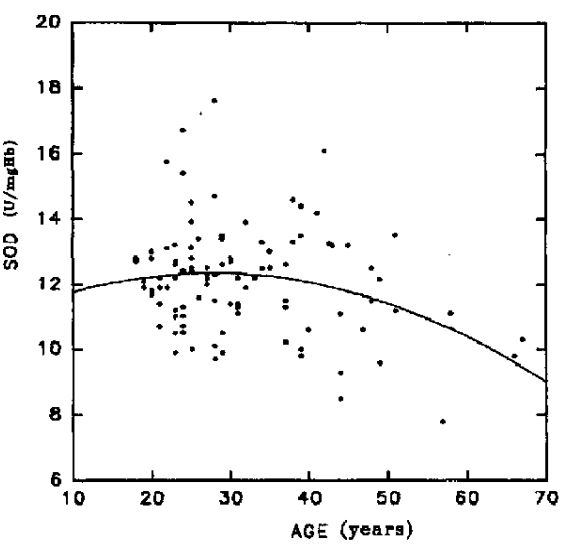

Figure 1 — Regression of age on SOD activity in human blood. Filled circles represent mean values of enzyme activity for each sample studied. Regression equation: ($Y = 10.94 + 0.102X - 0.002X^2$) $n = 103$.

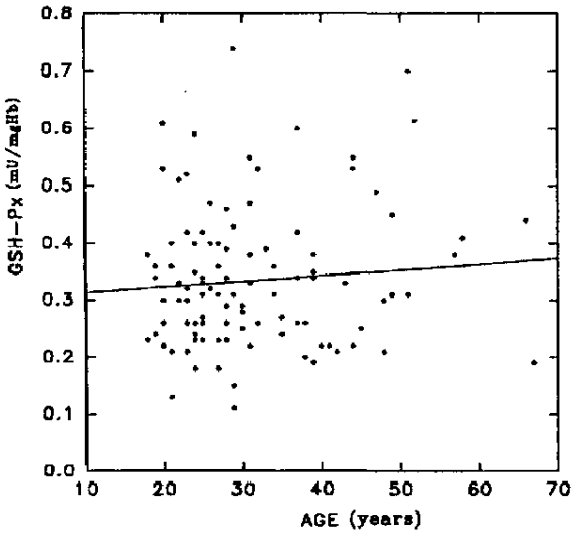

Figure 2 — Regression of age on GSH-Px activity in human blood. Filled circles represent mean values of enzyme activity for each sample studied. Regression equation: ($Y = 0.303 + 0.001X$) $n = 103$.

and GSH-Px in whole blood from healthy subjects, in order to obtain reference ranges for each AOE and analyze the influence of sex, age, and cigarette smoking on AOE activity. Our results are in good agreement with published data in that AOE activity in human blood exhibits a large interindividual variability (7,12–14,16,21). Recently, Lux and Naidoo (21) studied the biological variability of SOD and GSH-Px activities in the erythrocytes of 12 healthy subjects and reported an interindividual coefficient of variation of 16% for SOD and 27% for

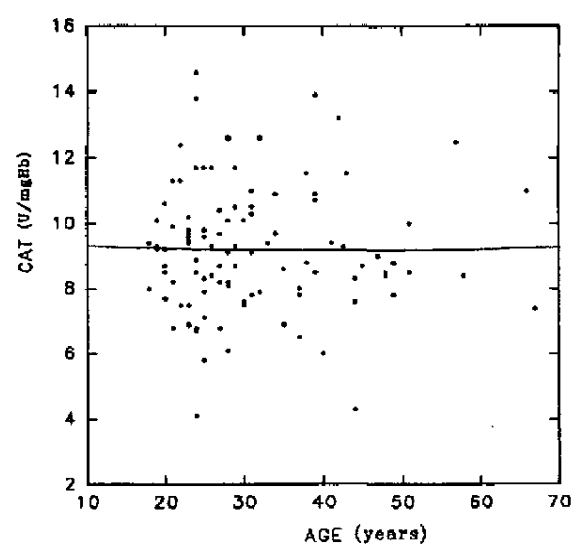

Figure 3 — Regression of age on CAT activity in human blood. Filled circles represent mean values of enzyme activity for each sample studied. Regression equation: ($Y = 9.22 - 0.001X$) $n = 103$.

GSH-Px. The interindividual coefficients of variation for the same AOE reported here (13.5% for SOD and 36.2% for GSH-Px) are in good agreement with the data of Lux and Naidoo (21) and confirm that GSH-Px exhibits the highest variability. The causes of this variability are at the present time not known. We explored the possibility that factors such as sex, age, or cigarette smoking may in part, play a causal role in the interindividual differences observed in AOE blood levels.

Our results show that females have higher levels of SOD and CAT and lower levels of GSH-Px than males. These findings are, at first sight, controversial with those from other authors who did not detect sex-related differences in SOD or GSH-Px activities in healthy subjects (15,16,20). However, these controversial findings may be partially due to differences in the enzymatic assays used and/or sample size. It is noteworthy to mention that all the studies regarding AOE activity in human blood from healthy subjects performed to date showed no differences or, as in the present one, slight differences between sexes. Hence, sample size may be a critical factor in determining the statistical significance of the differences observed. In fact, whereas Hopkins and Tudhope (15) and Winterbourn *et al.* (16) investigated GSH-Px and SOD activities in human blood using a sample of 52 and 62 subjects, respectively, in our present study and that of Ceballos-Picot *et al.* (20) over 100 samples were analyzed. Moreover, while the same authors (20) determined GSH-Px activity by using *tert*-butyl hydroperoxide, which allows the measurement of both seleno-dependent and non-seleno-dependent GSH-Px (total GSH-Px activity), we assessed the activity of this enzyme by using hydrogen peroxide, which allows the measurement of seleno-dependent GSH-Px only.

Recently, Ceballos-Picot *et al.* (20) detected a clear negative correlation between age and SOD levels in human erythrocytes. Our present finding of a linear quadratic effect of age on SOD activity in whole blood not only confirms that the activity of this enzyme in human blood decreases with age but also shows that this decrease is not linear, beginning at 28 years of age. Accordingly, a linear quadratic function describes more accurately the relationship between age and SOD activity. As suggested by Ceballos-Picot *et al.* (20), this age-dependent decrease may be due to a progressive enzyme inactivation by its product H_2O_2 (28,29) or due to an increase in the glycation of SOD (30). Clearly, further investigation is necessary to confirm these assumptions.

Moreover, we found a linear effect of age on seleno-dependent GSH-Px activity showing that the activity of this enzyme in blood increases with age. This finding agrees with that of Ceballos-Picot *et al.* (20) who found a positive correlation between age and total GSH-Px activity in human erythrocytes. However, Hopkins and Tudhope (15) found no apparent relationship of this sort. Unfortunately, the authors did not specify the statistical analysis used

TABLE 3
Influence of Cigarette Smoking on Antioxidant Enzymes Activity in Human Blood (mean ± SD)[a]

Gender	SOD (U/mgHb)	CAT (U/mgHb)	GSH-Px (mu/mgHb)
Males + Females (S,35)[b]	12.3 ± 1.7	9.0 ± 1.9	0.3 ± 0.1
Males + Females (NS,59)	12.1 ± 1.7	9.5 ± 1.9	0.3 ± 0.1
Males (S,17)	11.9 ± 1.9	8.5 ± 1.9	0.4 ± 0.1
Males (NS,29)	11.7 ± 1.2	8.3 ± 1.6	0.3 ± 0.1
Females (S,18)	12.7 ± 1.8	9.5 ± 1.8[c]	0.3 ± 0.1
Females (NS,30)	12.5 ± 2.0	10.6 ± 1.6	0.3 ± 0.1

SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase.
[a]The smoking status of 9 of the subjects was unknown.
[b]Smoking status and sample size: S = Smokers, NS = Nonsmokers.
[c]$p < 0.05$ compared with nonsmokers.

to determine that relationship, and thus, we cannot speculate about the reason of this disagreement. On the other hand, our investigation suggests that the activity of CAT in human blood is not affected by aging.

Even though we cannot assert that the observed sex- and age-related differences regarding AOE activity are of clinical interest, the fact that those differences are statistically significant suggests that for clinical purposes it may be necessary to take into account the sex and age of each of the subjects involved in a clinical study.

In summary, by using three simple and sensitive assays, suitable for clinical determinations, we confirm here that AOE activity in human blood exhibits a wide interindividual variability and we demonstrate that this variability may be ascribed, at least in part, to the sex and age of the individuals. Moreover, the finding that no significant differences were observed between smokers and non-smokers (except in the case of CAT activity in females) suggests that AOE activity in human blood is not affected by cigarette smoking, although this assumption requires further investigation to be confirmed.

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